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Microstimulation of the Lumbosacral Spinal Cord: Mapping

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ABSTRACT

The objectives of this project are to define the anatomical locations of spinal neuronal populations involved in control of genitourinary and motor functions, and to map the physiological responses evoked in the genitourinary and hindlimb motor systems by microstimulation in the lumbosacral spinal cord. During this quarter we have made progress on both of these objectives. We completed experiments to map the locations of spinal neurons involved in genitourinary function using expression of the immediate early gene c-fos, and completed cell counting to quantify the results from these experiments. The results have identified the location of spinal neurons involved in control of micturition and identified target regions for microstimulation. We have also begun using immunohistochemistry to determine the neurotransmitters that are present in the neurons labeled by c-fos. We continued microstimulation studies to map the genitourinary responses to microstimulation. Specifically, we expanded our mapping studies to include neurons around the central canal as these neurons were identified in the c-fos mapping studies as being active during reflex micturition. The results of these experiments suggest that microstimulation around the central canal can evoke voiding in the neurologically intact, chloralose anesthetized cat.

INTRODUCTION

Electrical stimulation of the nervous system is a means to restore function to individuals with neurological disorders. The objective of this project is to investigate the feasibility of neural prosthetics based on microstimulation of the spinal cord with penetrating electrodes. Specifically, we will use chemical and viral retrograde tracers, stimulation mapping, and field potential recordings to determine the locations in the spinal cord of the neuronal populations that control genitourinary and motor functions in the male cat. We will use selective microstimulation with penetrating activated iridium microelectrodes to determine the physiological effects of stimulation of different neural populations. The results of this project will answer fundamental questions about microstimulation of the spinal cord, and lead to development of a new generation of neural prosthetics for individuals with neurological impairments.

During the ninth quarter of this contract we continued anatomical mapping of spinal neurons active during micturition and began to determine their neurochemical identity. We also measured the pressures generated in the bladder and urethra by microstimulation around the central canal in the sacral spinal cord. This area was identified from our c-fos studies as being one containing neurons that are active during micturition. Below each of our accomplishments is summarized.

PROGRESS IN THIS QUARTER

I. Anatomical Tracing of Genitourinary Innervation

Expression of c-fos in Genitourinary-related Spinal Neurons

The purpose of these experiments is to determine the location and rostrocaudal extent of spinal neurons that participate in control of micturition. The immediate early gene c-*fos* that encodes the Fos protein can be induced rapidly and transiently in post-synaptic neurons by increased electrical activity [Chaudhuri, 1997]. In these studies we used expression of Fos protein to identify cells within the sacral spinal cord that regulate genitourinary function in male cats. During this quarter we completed quantification of the neurons expressing c-fos after a period of reflex isometric voiding (QPR 7). The results of these experiments identify neurons that were electrically active during the behavior of voiding, and thus identify spinal sites to target for microstimulation.

METHODS

The methods used to induce c-fos expression and to process the tissue have been described in our previous reports (QPRs 6-8). Here we describe the methods used to quantify the resulting cell labeling.

Quantification of the Distributions of Fos-positive Neurons

During this quarter we counted the number of cells exhibiting Fos-like immunoreactivity after periods of reflex isometric micturition and in an operated, unstimulated control. Slides were viewed using bright-field microscopy and locations of cells exhibiting Fos-like immunoreactivity described according to the conventions of Rexed [1954]. High resolution, low magnification prints were made of 3 sections from each segment (S1, S2, S3, L3, L4) in each animal. The printouts were used to mark Fos-positive cells identified under higher magnifications views of the same sections. The number of cells exhibiting Fos-like immunoreactivity in each lamina were averaged across 3 sections from each segment in each animal. All cell counts were made by an investigator blinded to the procedure conducted on the animal.

RESULTS

In all animals undergoing isometric micturition, nuclei exhibiting Fos-like immunoreactivity (FLI) were found bilaterally in S1-S3. Few neurons, restricted to the superficial dorsal horn were observed in the control case, while 1-4 h. of isometric micturition generated FLI in neurons in the superficial dorsal horn, around the central canal, and within the intermediolateral region. Fewer Fos-immunoreactive nuclei were observed in the medial portion of the superficial dorsal horn, and FLI was not observed in ventral horn neurons. Examples of neurons expressing FLI can be seen in figure 9.3.

Influence of Duration of Reflex Micturition and Anesthetics

An analysis of variance was conducted to determine whether the duration of isometric micturition or type of anesthesia had an effect on the expression of FLI in the spinal cord, and a post-hoc comparison between pairs made using Tukey's test. Although there were no apparent differences in the spatial patterns of FLI generated in the spinal cord by 1, 2, or 4 hours of isometric micturition, there were quantitative differences in the numbers of labeled cells. Figure 9.1 shows the numbers of neurons expressing FLI in each of the three spinal regions: 1. dorsal horn, 2. intermediolateral region, and 3. around the central canal, under different experimental conditions. Data are the average of cell counts over 3 samples from each of the spinal segments in 6 animals undergoing isometric micturition and an operated, unstimulated control. The control animal exhibited few cells which were primarily localized to the dorsal horn (region 1). In the experimental animals the mean number of cells per section was higher after 1 hour of isometric micturition (18±15 cells, mean±s.d.) than after 2 hours (8±10 cells) or 4 hours (11±10 cells) of isometric micturition ($p<0.01$), but there was no difference between the number of cells per section after 2 hours and 4 hours ($p=0.13$). Similarly, there were no apparent differences in the spatial patterns of FLI generated in the spinal cord in animals receiving ketamine and the animal not receiving ketamine (i.e., animals receiving halothane), and although the mean number of cells per section was higher in the halothane animals (12±13 cells) than in the ketamine animals (9±10 cells), this difference was not significant ($p=0.58$).

Sacral Spinal Neurons Expressing c-Fos after Isometric Micturition

Figure 9.2 shows quantitatively the laminar distribution of neurons expressing FLI at each of the sacral segments. Each bar shows the mean and standard deviation from cell counts in 3 sections from each segment in each of 5 animals undergoing fictive micturition. The 1 hour animal was excluded from this group because of the differences noted above. In the animals undergoing isometric micturition, Fos-positive neurons in the sacral segments were found in the superficial dorsal horn in lamina I, predominantly in the lateral aspect, and a smaller number of cells were present in the lateral aspect of lamina II. Large numbers of neurons expressing FLI were observed in the intermediolateral region consisting of the lateral parts of laminae V-VII, particularly in the S2 segment, and fewer Fos-positive neurons were present in the medial parts of these laminae. Neurons expressing FLI were also found around the central canal in lamina X across all three

sacral segments with the largest numbers present in S2. Fewer cells were found in the lumbar (L3-L4) spinal segments than in the sacral spinal segments. In the lumbar segments most cells were observed in the superficial dorsal horn (lamina I, II). Fos-positive neurons were also observed in the intermediolateral region (lateral laminae V-VII), as well as in the medial part of lamina V.

The locations of neurons expressing FLI parallel areas where terminals of primary afferents from the pelvic nerve have been documented, including the dorsal commissure, the intermediolateral region, and the lateral dorsal horn [Morgan et al., 1981, Nadelhaft and Booth, 1984]. Similarly, primary afferent terminals from the pudendal nerve project to the dorsal commissural gray, the superficial layers of the dorsal horn, and to the dorsal aspect of the intermediolateral region [Ueyama et al., 1984]. The locations of Fos-positive neurons after isometric micturition is also consistent with electrophysiological studies demonstrating that neurons in the sacral parasympathetic nucleus (SPN) and around the central canal receive excitatory inputs from both somatic and visceral afferents [de Groat et al., 1981, McMahon and Morrison, 1982, Honda, 1985].

Co-localization of Neurotransmitter Expression in c-fos Expressing Neurons

During this quarter we began a set of studies to identify the neurochemical identity of neurons in the sacral spinal cord. The work described above and in previous progress reports enables identification of the location of neurons involved in regulation of genitourinary function. However, these data do not provide any information on the function of the identified neurons. As a first step in identifying neural function we are co-localizing neurotransmitters with c-fos in genitourinary related neurons. During this quarter we used immunohistochemistry to localize neurons expressing γ-aminobutyric acid, an inhibitory transmitter with widespread distribution in the spinal cord. To locate GABAergic neurons, we used a primary antibody to parvalbumin in tissue sections from the same animals in which neurons active during micturition were visualized with c-fos (see above). Parvalbumin is a calcium binding protein that is found in GABAergic neurons of the central nervous system [Heizmann, 1984, Celio, 1986].

METHODS

Sections of the sacral spinal cord taken from animals which had undergone 2 hours of isometric micturition were double-processed using immunohistochemistry to detect the presence of c-Fos and GABA by detection of parvalbumin. The free floating sections were washed in PBS containing 0.3% Triton-X and then exposed for 30 min to PBS-Triton solution containing 3% normal rabbit serum to block non-specific binding sites. After a further wash, the tissue was placed overnight at room temperature in a primary polyclonal antibody solution (1:10,000 dilution of rabbit anti-Fos (Ab-5) in PBS; Oncogene, Cambridge, MA). The sections were rinsed, incubated with biotinylated goat anti-rabbit secondary antiserum and further processed using the standard biotin avidin-peroxidase kit (ABC-elite kit; Vector Labs, Burlingame, CA). The immunoreaction was visualized by incubating the sections with 0.02% 3.3-diaminobenzidine containing 0.01% hydrogen peroxide for 6 min. A purple-black reaction product was obtained by adding nickel chloride to the peroxidase reaction (40 ml of 8% $NiCl_2$ solution per 100 ml of DAB solution). Thereafter, sections were incubated for 16 hours in a solution containing monoclonal antibody formed in mouse (1:2,000 mouse anti-parvalbumin (PARV-19) in PBS; Sigma, St. Louis, MO). The sections were washed in PBS (2x), incubated for four hours with biotinylated goat anti-mouse secondary antiserum, and further processed using the standard biotin avidin-peroxidase kit (ABC-elite kit; Vector Labs, Burlingame, CA). Parvalbumin immunoreactivity was visualized by the peroxidase reaction omitting the $NiCl_2$ addition to DAB to obtain a brown reaction product.

RESULTS

Co-localization of Fos-immunoreactivity with parvalbumin-immunoreactivity was identified by viewing the sections under bright field. The contrasting immunoprecipitates were readily distinguishable. Immunostaining for Fos protein was exclusively nuclear, and immunoreactive nuclei were seen as dark, round or oval structures (fig. 9.3). Immunoprecipitate indicating parvalbumin immunoreactivity was confined to the cytoplasm. Parvalbumin (PV) containing cells were observed in the sacral spinal cord in regions where Fos-containing cells were also found including around the central canal and within the intermediolateral region. These PV-containing cells were of medium-size and included both stellate and spindle varieties. PV-containing cells in the ventral horn had large somas, centrally located nuclei, and well stained processes. Control experiments were done to determine whether the primary or the secondary antibodies produced false positive results. Omission of primary or secondary antibodies resulted in absence of labeling, demonstrating that no false positive results were obtained with these reagents.

Figure 9.3 shows a group of cells around the central canal in the S2 segment that are stained for parvalbumin. These results indicate that these neurons express the inhibitory neurotransmitter GABA. This figure also shows a group of neurons that expressed c-fos after a period of isometric micturition. Interestingly, parvalbumin was not found to co-localize with c-fos.

Previous results indicate that neurons around the central canal in the sacral cord, that receive descending input from Barrington's nucleus, contain the inhibitory neurotransmitter GABA [Blok and Holstege, 1997]. Furthermore, external urethral sphincter motoneurons are contacted by GABAergic terminals [Ramirez-Leon and Ulfhake, 1993, Ramirez-Leon et al., 1994]. Therefore, we expected to find GABA co-localized with c-fos in some spinal neurons active during micturition. However, while all parvalbumin-positive neurons are also GABAergic, not all GABAergic neurons are parvalbumin positive [Cowan et al., 1990]. Therefore, our result must be confirmed with a more specific antibody such as anti-GABA or anti-GAD (glutamate decarboxylase, a GABA precursor), before concluding that none of the c-fos positive neurons express GABA.

II. Microstimulation of the Sacral Spinal Cord in Male Cats

The objective of these experiments is to determine the physiological effects in the genitourinary system of microstimulation of neuronal populations in the spinal cord. The specific focus of current work was to measure bladder and urethral pressures produced by microstimulation around the central canal. As described above, neurons around the central canal are active during reflex micturition and receive descending connections from Barrington's nucleus. Therefore, the effects of stimulating these neurons was of interest to produce micturition.

Methods

Male cats were sedated with xylazine (2 mg/kg), anesthetized with ketamine HCl (15 mg/kg, IM), and a venous catheter was inserted in the cephalic vein. Anesthesia was maintained with α-chloralose (60 mg/kg IV, supplemented at 15 mg/kg). A midline abdominal incision was made to expose the bladder, the ureters were tied, transected and drained externally, and the bladder was cannulated with a PE tube. A laminectomy was made from L4 to S1 to expose the sacral cord. The animal was mounted in a spinal frame with pins at the hips, the head in a headholder, and a vertebral clamp at L3. Body temperature was maintained between 37° and 39° C with a heat lamp, warm 5% dextrose saline with 8.4 mg/cc sodium bicarbonate added was administered IV (~20 cc/hr), and the animal was respired artificially.

The pressures generated in the bladder were measured using a solid state pressure transducer connected to the suprapubic PE catheter (Deltran DPT-100, Utah Medical Products, Midvale, UT). The bladder volume was set to a level (5-10 cc) that was below the threshold to induce reflex contractions of the bladder. Urethral pressures were measured using a custom 2-channel micromanometer (MMI, Hackensack, NJ) which has 2 directionally sensitive elements

spaced 3 cm apart and circumferentially offset by 180°. The catheter was used to measure simultaneously the pressure in the sphincteric (6 cm from external meatus) and penile (3 cm from external meatus) urethra. The pressure signals were amplified, low pass filtered (fc=30 Hz), and continuously recorded on a strip chart recorder. Pressures evoked by stimulation were also sampled by computer (fs=100Hz).

Stimulation was accomplished with activated iridium microwire electrodes (50μm Epoxylite insulated iridium wire with an exposed electrochemically determined surface area of ~225 μm^2 and a 1 μm tip, HMRI IS300, Huntington Medical Research Institutes, Pasadena, CA). Stimuli were charge balanced biphasic pulses with an amplitude of 10-150μA and a pulsewidth of 100μs applied as 1 s to 100 s continuous trains with a frequency between 2 Hz and 100 Hz. The standard mapping stimulus was a 1 s 20 Hz train of 100 μA 100 μs pulses.

The individual dorsal and ventral roots were stimulated using a hook electrode and functionally identified. The root that produced the largest bladder pressure was presumed S2, and this identification was confirmed by stimulation of other roots rostral and caudal to the putative S2 root and post-mortem dissection. Vertical, dorsal-to-ventral penetrations (increment=100-400 μm) were made at multiple mediolateral locations (increment=250 μm) in the middle of the S2 segment, at the S1-S2 border, and at the middle of the S1 segment.

Results

As expected from the results of our c-fos mapping experiments, microstimulation in regions around the central canal generated responses in the genitourinary system. These responses included selective contraction of the bladder, selective contraction of the periurethral striated musculature, as well as co-contraction of the bladder and periurethral striated muscles.

The bladder pressures, proximal urethral pressures (~6 cm from external meatus), and distal urethral pressures (~3 cm from external meatus) evoked along tracks traversing the pericanicular gray in the S2 and S1 spinal segments are shown in fig. 9.4. The two parallel tracks in the mid-S2 segment in fig. 9.4A and 9.4B illustrate that there is a region ventrolateral to the central canal where microstimulation generated bladder pressures without increases in urethral pressure. At more ventral locations, pressures were evoked in both the proximal and distal urethra, but not on the bladder.

Figure 9.4C shows that in the S1 segment microstimulation in the same region also generated increases in bladder pressure without increases in urethral pressure. In this case the pressures were much higher than those evoked in the S2 segment (30+ cmH_2O *vs.* 5 cm H_2O). At more ventral locations microstimulation generated co-activation of the bladder and proximal and distal urethra.

Figures 9.4D and 9.4E show individual responses at 2400 μm below the surface in the S1 segment (i.e., lateral to the central canal, fig. 9.4 C). The responses to the 1 s 20 Hz mapping stimulus illustrate an increase in bladder pressure, a weak increase (~2 cmH_2O) in proximal urethral pressure followed by transmitted pressure from the bladder, and a slight decreases in pressure in the distal urethra. These responses prompted testing of the responses at this location to longer duration stimuli, as required for functional responses. The responses at the same location generated by a train duration of 100 seconds are shown in fig. 9.4D. A large, sustained increase in bladder pressure was observed, and identical pressure responses were observed in the proximal and distal urethra. This indicates that the three sensors were equibaric and thus were connected by a fluid column. During this train the animal voided approximately 2 cc out of 7 cc in the bladder, even though the urethral catheter was in place.

These results demonstrate that microstimulation in regions around the central canal produce responses appropriate for generating micturition by microstimulation. Similar results have been reported recently by other investigators [Woodford et al., 1996, Blok and Holstege, 1997]. In must be noted that these responses were obtained in neurologically intact, anesthetized animals, and should be verified in spinal animals.

OBJECTIVES FOR THE NEXT QUARTER

I. Anatomical Tracing of the Genitourinary and Hindlimb Motor Systems. During the next quarter we will prepare and submit a manuscript reporting the results of the c-fos mapping studies. We will continue our co-localization studies to identify the neurotransmitters present in neurons active during micturition and submit an abstract to the Society for Neuroscience Meeting reporting our results. Finally, We will initiate a study using spinally injected retrograde tracers to map the location and rostrocaudal extent of last order interneurons that project to spinal motoneuron pools.

II. Microstimulation of the Lumbosacral Spinal Cord: During the next quarter we will prepare and submit a manuscript reporting the results on our studies on the hindlimb motor responses generated by microstimulation of the lumbar spinal cord. We will continue experiments to map systematically the genitourinary responses generated by microstimulation of the sacral spinal cord, with an emphasis on regions around the central canal.

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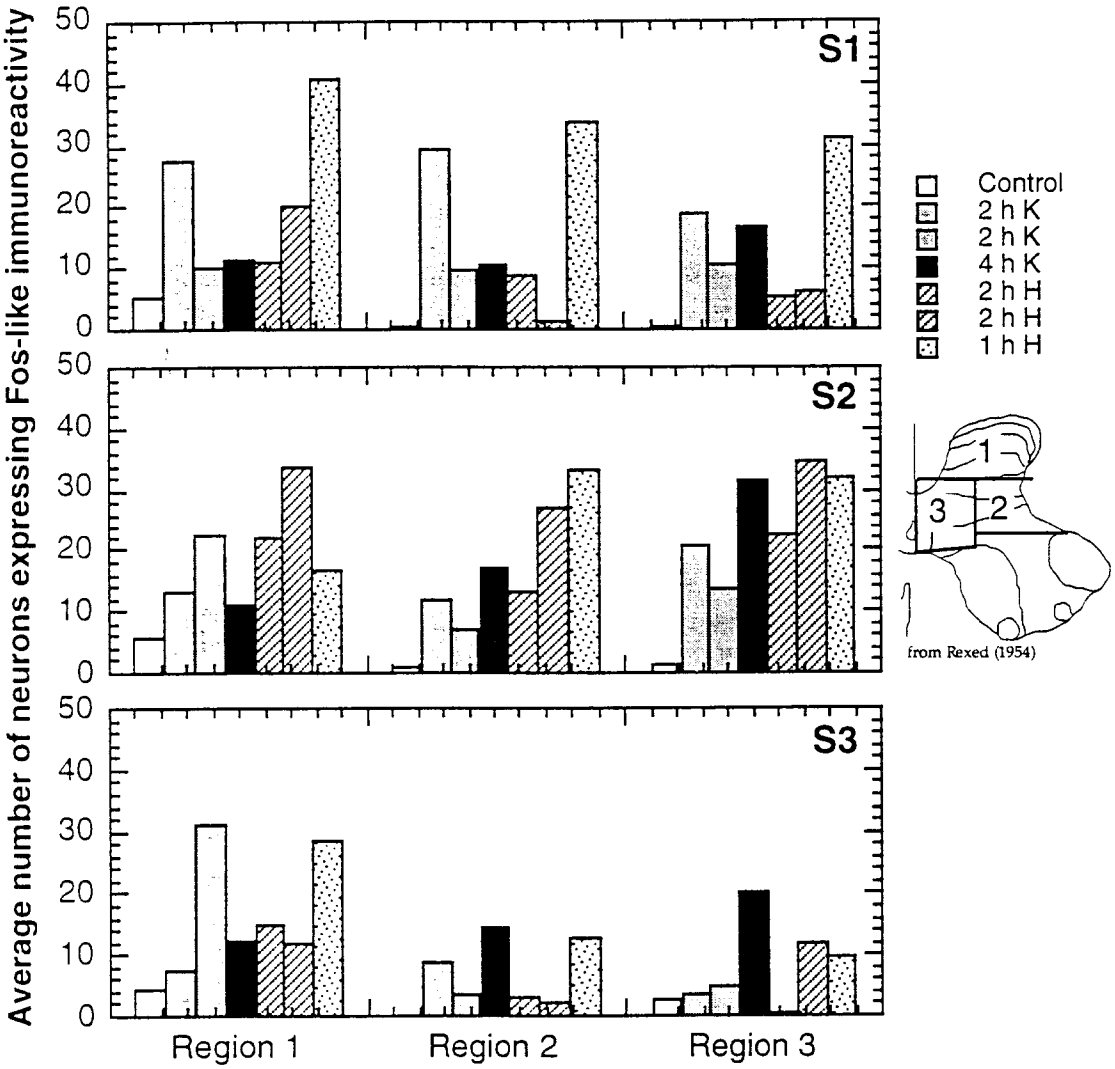

Figure 9.1: Influence of duration of isometric micturition and anesthesia on the pattern and intensity of spinal c-Fos expression. Histograms of the numbers of expressing Fos-like immunoreactivity in each of the three spinal regions (see inset at bottom): 1. laminae I-IV, 2. lateral laminae V-VII, and 3. medial laminae V-VII plus lamina X. Each bar represent the average of cell counts over 3 sections from each of the spinal segments examined. Note the change in ordinate scale for the lumbar regions.

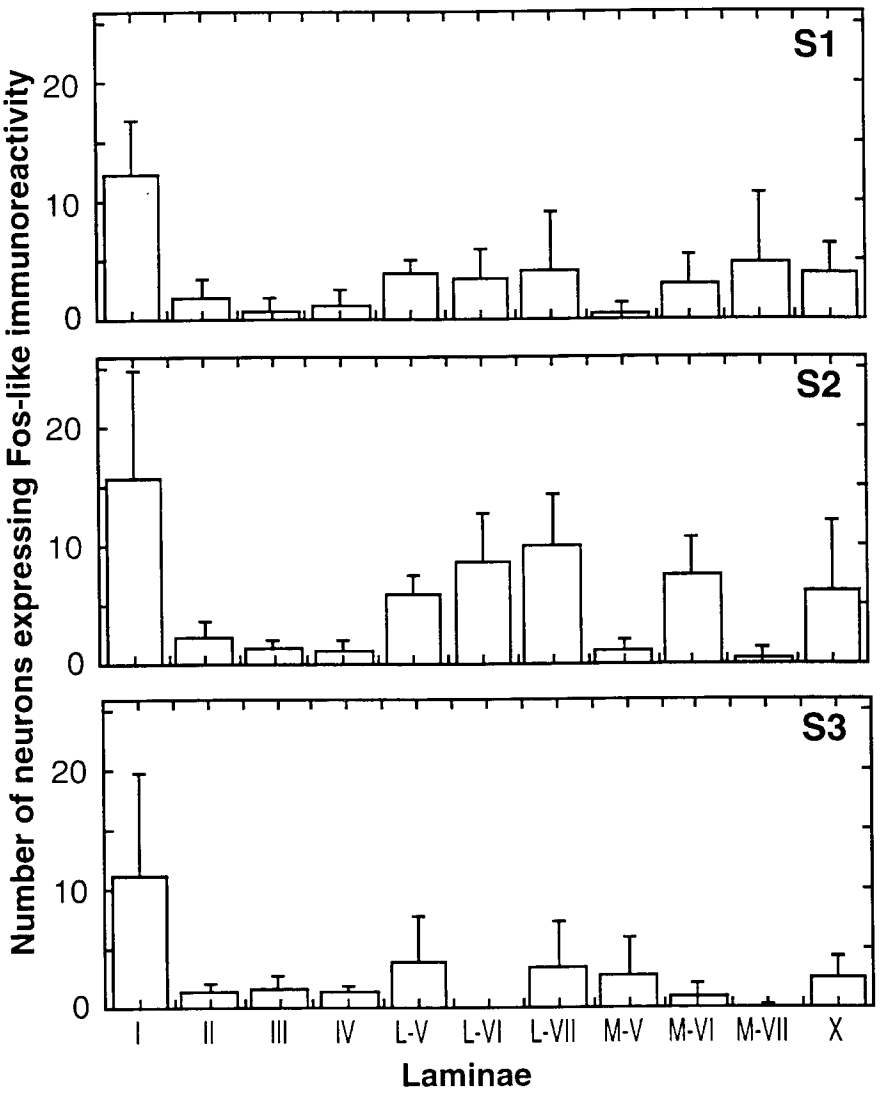

Figure 9.2: Quantification of patterns of spinal c-Fos expression induced by isometric micturition. Histograms of the laminar distribution of neurons expressing Fos-like immunoreactivity at each of the sacral spinal segments. Lamina V, VI, and VII were separated into medial and lateral regions. Each bar shows the mean and standard deviation of the number of cells counted in three sections in each of 5 animals undergoing 2 hours or 4 hours of isometric micturition.

Figure 9.3 Immunohistochemical identification of neurotransmitters in the cat sacral spinal cord. Photomicrograph of co-localization of c-fos and neurotransmitter expression in neurons of the sacral spinal cord in male cats. Neurons near the central canal in the S2 spinal segment stained for c-fos and parvalbumin (PV). Fos-immunoreactive neurons are identified by their round to oval dark nuclei (arrowheads), while PV-immunoreactive neurons are identified by their dark cytoplasm (arrows). PV and c-fos were not found in the same cells as seen clearly in the 2 cells in this section (open arrows) with nuclei not expressing c-fos (i.e., clear) but with PV clearly present in the cytoplasm. Bar = 100 μm.

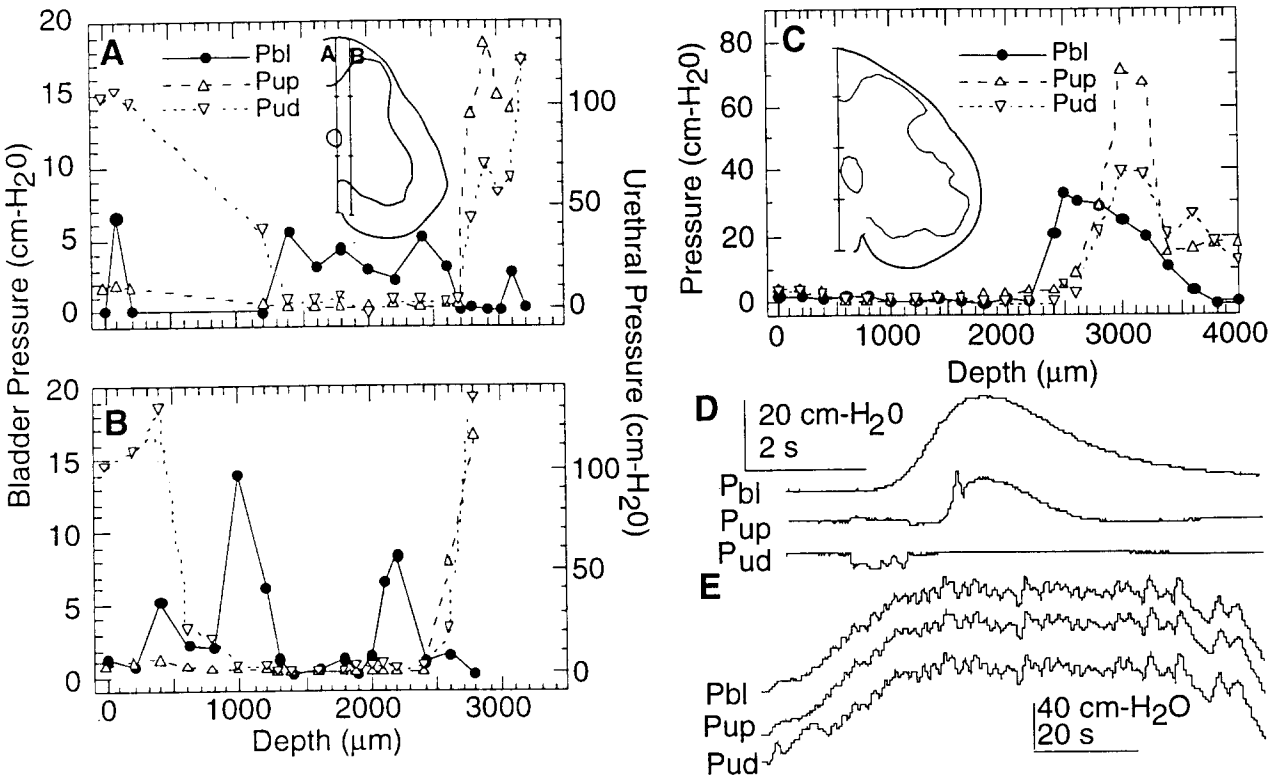

Figure 9.4: Pressures evoked in the bladder (Pbl), proximal urethra (Pup; 6 cm from external meatus), and distal urethra (Pud; 3 cm from meatus) evoked by microstimulation of the pericanicular gray in the sacral spinal cord. A, B.) Genitourinary responses generated by microstimulation along 2 penetrations through the S2 segment. Stimulus was a 1 s 20 Hz train of 100 μs, 100 μA pulses. The inset in A shows a tracing of the right S2 spinal cord and the locations of the two penetrations (1000 μm per division). C.) Pressures evoked in the bladder (Pbl), proximal urethra (Pup; 6 cm from external meatus), and distal urethra (Pud; 3 cm from meatus) by microstimulation along a penetration through the S1 segment. Stimulus was a 1 s 20 Hz train of 100 μs, 100 μA pulses. The inset shows a tracing of the right S1 spinal cord and the locations of the penetration (1000 μm per division). D, E.) Traces of pressures evoked in the bladder (Pbl), proximal urethra (Pup), and distal urethra (Pud) by microstimulation (bar) at 2400 μm in C with either a 1 s (D) or 100 s (E) 20 Hz train of 100 μA 100μs pulses. The insets in A and C show tracings of the right S2 and S1 spinal cords and the locations of the penetrations (1000 μm per division).